BROWN, WILLIAM

STUDIES IN THE PHYSIOLOGY OF PARASITISM

I. THE ACTION OF BOTRYTIS CINEREA

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Studies in the Physiology of Parasitism.[1]

I. The Action of Botrytis cinerea.

BY

WILLIAM BROWN, M.A., B.Sc.

CONTENTS.

A. INTRODUCTION.

THE physiological relation of host and parasite is a subject urgently in need of investigation at the present time. On a knowledge of such relations must depend any further insight into the nature of immunity and susceptibility of plants to disease. In the case of the more highly specialized parasites there is considerable difficulty in studying their physiological relations to their host owing to the complexity of the relationship, which often amounts almost to symbiosis, and to the difficulty of growing the parasites independently and of bringing about infection at all times of the year. It seemed probable, then, that a closer investigation, by modern biochemical methods, of the mode of action and method of infection of some of the simpler parasites which can be easily cultivated would be of great value. A knowledge of the relationship of such forms to their hosts should lead the way to a better understanding of the more highly specialized parasites. It was with this view that the present investigation was undertaken.

[1] This is the first of a series of studies which are being carried out in the department of Plant Physiology and Pathology of the Imperial College of Science and Technology.

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Botrytis cinerea is a member of the large physiological group of Fungi known as 'Facultative Parasites', the members of which while normally saprophytic are able under certain conditions to live parasitically. Among Fungi of this type, the subject of the present investigation is somewhat pre-eminent, partly on account of its ubiquity, partly by reason of the striking features of its parasitic attack. When to these is added the fact of its being very amenable to cultural treatment, it is not surprising that this form has become a classical one for the study of the physiological questions relative to this type of parasitism. As a result of these researches certain features of this type of parasitic activity may now be considered as being firmly established. Prominent among these is the one due to de Bary (1), and since his day frequently confirmed, that such Fungi possess the power of killing and disintegrating the tissues of the host in advance of their growth, so that properly speaking they are not parasitic upon the living host at all, but live merely on its dead remains. This phenomenon of 'action in advance' de Bary set down to the excretion by the tips of the advancing hyphae of some soluble substance which is capable of bringing about the observed changes—changes consisting, firstly, in the solution of the cell-wall, or at least of certain of its constituents, so that the tissue loses its coherence; and, secondly, in the killing of the living protoplasmic contents themselves. So far we find general agreement, but when we come to treat of the nature of this soluble substance, of what it consists, of how many chemical entities it consists, and what share in the process is assignable to each, we meet with considerable divergence of opinion. This will be made clear from a review of the literature dealing with this question.

B. Historical.

De Bary (1), in a paper now classical, investigated the parasitism of *Sclerotinia Libertiana*, a form closely allied morphologically to *Botrytis cinerea*, and showing many similarities in its mode of parasitism. By using the expressed juice of certain plant organs (roots of carrot, &c.) which had been infected and overrun by the fungus, as also the liquid exuded in droplets from the young sclerotia of the fungus itself, he was able to show that the active principle was thermolabile, and so concluded that it was of the nature of a ferment. The cell-wall dissolving activity of the fungus he unhesitatingly ascribed to this enzyme; as to the toxic activity he was somewhat doubtful. He discussed the possibility of the latter being due to some soluble oxalate, and while showing that oxalates do occur in certain secretions of the fungus, he was unable on quantitative grounds to ascribe to these the markedly destructive effects of the fungus. His final conclusions may be taken as summarized in the following quotation: 'The difference (between boiled and unboiled extract) is indeed a quantitative one as far as can be observed. . . . With the liquid from sclerotia the same

differences appear, though less prominently—the boiled liquid has here a relatively greater effect.'

Marshall Ward (2), in his treatment of this problem, made flask cultures of *Botrytis* on prune juice. After the lapse of three weeks, he removed the superficial weft of mycelium, which was then washed, dried, ground to a powder, and extracted in water. He was able to confirm de Bary as regards the presence of a cell-wall dissolving enzyme ; as regards the toxic nature of the extract he was unable to make any advance.

Precisely similar results had been obtained a short time previously by Kissling (3), the same type of method being employed.

Nordhausen (4) strongly emphasized the twofold aspect of the phenomenon, enzymic and toxic. The following represents his conclusions : the active substances are 'apparently in part likewise enzymes, a conclusion which I reach from experiments similar to those of de Bary, although it is not excluded that oxalic acid under certain circumstances plays a part. That the latter, however, is not alone responsible for the toxic action on plant tissue is shown by the case of *Aspergillus niger*, which secretes oxalic acid in large quantities, even more so than *Botrytis*, without showing in any similar degree the marked parasitism of the latter.'

Behrens (5) used the expressed juice of fruits which had been infected with certain Fungi ; thus in one case that of a pear infected with *Mucor stolonifer*, in another that of an apple infected with *Penicillium luteum*. In the latter case he mentions that the culture was allowed to develop for three months before the extract was investigated. These juices he found to be toxic to the mesocarp cells of *Symphoricarpus* berries, nor were the toxic effects reduced by boiling. Hence he concluded that the toxic substance was neither of volatile nor of enzymatic nature.

Smith (6) compared the actions of weak oxalic acid solutions (0·01 per cent. to 1 per cent.) and of a mycelial extract of *Botrytis* on certain plant tissues (stem of lettuce), and while noting that the effects were not similar in all particulars—especially in respect of the post-mortem reactions induced—he concluded that oxalic acid, even in the lowest concentrations employed, was sufficient to produce the changes observed. He even goes so far as to state, on the basis of certain maceration effects of the acid, that the cell-wall dissolving effects of the fungus may be set down to this acid, quoting from de Bary that so much as 0·3 per cent. oxalic acid may occur in mycelial extracts. This author thus occupies an extreme position among those who ascribe an agency in this question to oxalic acid.

As papers cognate to this subject may be cited those of Jones (7), Potter (8), and Van Hall (9). These investigations refer to bacterial plant diseases, and in each case it was shown that a cell-wall dissolving ferment could be obtained from media in which the organisms had been cultivated. This ferment is no doubt very similar to the *Botrytis* ferment under

consideration. On the question as to the presence of the toxin these authors were unable to make any notable contribution.

Criticism. Viewed generally, the methods of preparing the fungal extract which have been adopted by previous workers fall under the two heads:

1. *Extraction from the old mycelium.* This is the method of de Bary, Marshall Ward, Kissling, Smith, and Nordhausen.

With respect to this method it can reasonably be objected that the secretions of an old mycelium do not necessarily bear any close relationship to those of a young vigorous culture. It is to be borne in mind that the active invading portion of the fungus is essentially of the nature of a young and fresh culture. In order therefore to study the nature of the active principle of the fungus, it is necessary to examine the secretions of young and vigorously growing hyphae. In this way only can the complications due to the presence of waste products ('staling' products) be avoided.

2. *Extraction from plant organs* (fruits, tubers, &c.) which have been overrun by the fungus. This method was employed by de Bary, and more particularly by Behrens.

The time required for the complete invasion of a compact structure such as a good-sized fruit may be considerable; in one case, for instance, Behrens employed a three months' old culture of *Penicillium luteum* on apple. It will thus be seen that the objections relative to old and (in the main) stale cultures put forward under the previous heading apply here with equal force. Furthermore, this method presents additional complications. In extracts of this sort there are present substances derived both from the fungus and from the host, in varying amount according to the degree of invasion at the time of extraction. Now when we bear in mind the plasmolysing effect of plant and especially of fruit juices, and furthermore that plasmolysis normally induces death of living tissue, it is easy to see that such extracts as those of de Bary and Behrens can give no certain indication of the primary toxic principle concerned. It will be shown later that the plasmolysing effect of a plant juice is greatly reduced by the growth in it of a fungus. Nevertheless the presence of the unknown remainder constitutes in all cases a complication which certainly should be avoided.

A similar criticism would also apply to the bacteriological investigations above mentioned. Here, however, the active solutions obtained were so weak that little was attempted in the way of studying the question of toxicity.

The work of Smith calls for special criticism. His method was to compare the action of mycelial extract with that of various strengths of oxalic acid, and from certain similarities he concluded that this acid played a great part in the changes concerned. Other acids he found behaved

in this respect like oxalic acid; he chose the latter from its known occurrence in the fungus. In one important particular, however, he is at fault, inasmuch as the starting-point of his study is a strange misquotation from de Bary. The latter he cites for the statement that oxalic acid of 0·3 per cent. strength may be found in extracts of *Sclerotinia*, whereas de Bary states categorically that he was unable to demonstrate any *free* oxalic acid, but that this substance occurs solely in the form of its salts (potassium or calcium). Smith himself records 2 per cent. oxalic acid in extracts of old mycelia, but here he is undoubtedly including oxalates, both soluble and insoluble. In the low concentrations employed by Smith, potassium oxalate has only a very slow toxic action, and its macerating action is nil at all concentrations. Such being the case, it cannot be allowed that Smith's contributions on this particular question are really helpful.

Apart from these considerations, a perusal of the literature convinces one that hitherto no one has succeeded in obtaining a really strong solution of the active principle of *Botrytis cinerea* or of any of its near allies. This we may conclude from the times required to bring about the various decompositions as recorded by the different investigators. Thus de Bary and Marshall Ward, in testing the action of their extracts on thin sections of plant tissue under the microscope, speak of a definite change being observable after some hours, while in the case of Behrens the action of the extract was allowed in some cases to proceed for twenty-four hours before observation was made. Now as regards the toxic action of an extract, it is absolutely imperative that the transformations be carried out within a comparatively short time. In work of this description—where the behaviour of living tissue is used as the indicator of a reaction—the complete exclusion of micro-organisms is a practical impossibility, and therefore observation can only be extended with confidence over the period during which bacterial activity is inconsiderable and while there is no accumulation of bacterial excretory products. How long this period may be will depend on various factors, prominent among which is the temperature maintained; but, speaking generally, it has been found that under ordinary laboratory conditions it is only with the help of special control measures that observation can be safely extended up to and beyond twenty-four hours from the commencement of the reaction. It is possible to extend the period of observation by lowering the temperature, a procedure which greatly retards the progress of bacterial contamination, without retarding in anything like the same degree the activity of the extract. Needless to say the employment of antiseptics is not permissible in studies of the toxic action of an extract.

The aim, then, of the present investigation was in the first place to prepare an extract from young hyphae alone, and it was hoped that this extract might prove to be of a sufficiently powerful nature to

recommend it for use in such an investigation as it was intended to prosecute. This anticipation has not been disappointed. It was found possible to obtain from young recently germinated spores of *Botrytis* an extract much more powerful than any hitherto attained, and furthermore a method has been elaborated for obtaining such material in fairly large quantities without prohibitive labour. As the method, though containing nothing new in principle, is novel, insomuch as no such procedure has hitherto been attempted, and as it may be found to be applicable to other studies along similar lines, it will be described in some detail.

C. Preparation of Standard Extract.

As it was thought advisable to use the same strain of fungus throughout, certain cultural precautions were taken in order to retain its vigour, and more especially its capacity for spore production. Up to the present time two primary cultures of *Botrytis cinerea* have been employed, one in the earlier stages, the other throughout the remainder and greater part of the investigation.[1] The former strain ceased after a few months to produce spores in abundance, and no treatment, such as alteration of temperature or of medium, was effective in restoring it to its original freely sporing condition. Up to this stage the practice had been to reculture the stock cultures rather frequently—once a fortnight—and to incubate them at 25° C. With the new culture this procedure was modified, stock cultures being kept at laboratory temperature (about 20° C.), and only undergoing reculture at much longer intervals—actually once in about three months. The present strain has now been in culture in this laboratory for a year and a half, in which time it has reached its sixth generation, and it still spores as freely as ever. Stock cultures are made in large 'boiling tubes' on the potato agar medium to be described below.

The first object is to provide an abundant supply of spores. This is done by the usual Petri-dish method, the culture medium being inoculated with the spores prior to pouring in order to obtain uniformity of development over the plate. As culture media, trials were made of turnip, prune, and potato agars. From the point of view of copious spore formation, the last is incomparably the best, and it has accordingly been employed throughout. It has the following composition:

Peeled potato	200 grs.
Agar	10 grs.
Water	to 1 litre.

The potatoes are boiled in water till they form a mush, which after cooling is pressed through a muslin bag in order to break down the larger

[1] Both cultures were obtained from the 'Centralstelle für Pilzkulturen', Amsterdam.

lumps. The subsequent treatment is as usual. Plate cultures are incubated at 20° C. (at 15° the spore yield is very much diminished). Spore formation begins in about 4 days; in 10 days to a fortnight the plates are ready for the subsequent treatment.

The problem now is to obtain the spores from the plate cultures. For this purpose each plate is covered with a layer of distilled water; then by gentle rubbing with the finger, beginning at the centre and working to the margin, it is possible to expel the entangled air without at the same time unduly contaminating the atmosphere of the room with spores, though this is unavoidable to some extent. The whole aerial portion of the culture—mycelium and spores—is now rubbed off by gentle scraping with a blunt knife. With a little practice it is quite easy to perform this operation without disturbing the underlying solid medium, so that, apart from the fungus itself, only liquid substances are removed from the plate. The fungal débris, &c., is now filtered through a fine clean muslin cloth (20 threads to the cm.). The spores and finer particles pass through, whereas the general mycelium, apart from occasional very short pieces of hyphae, is completely held back. The spore suspension is now centrifuged at a moderate speed. By this means it is possible to separate the heavy spores which are readily thrown down from the finer débris which remains in suspension. The 'wet' volume of the centrifuged spores is noted for purposes of the following treatment.

The centrifuged spores thus obtained in a practically pure form are now suspended in definite proportion in a nutrient liquid and spread over a glass plate in order to germinate. The following general considerations may be noted:

1. *Length of Period allowed for Germination.*

As it was proposed to examine the physiological conditions prevailing within the 'infection drop'[1] with the object of throwing some light on the manner in which the fungus first actually enters the host plant, an attempt was made to obtain an extract from the fungus at a stage comparable with that at which it actually penetrates the host. The period elapsing from sowing to penetration varies somewhat with different hosts and under different conditions, but generally speaking it may be set down as lying between 12 and 24 hours. Throughout this work the spores were allowed as nearly as might be 23 hours' germination, this being considered sufficiently close to the ideal time, as well as offering conveniences for the systematic day-by-day repetition of the process which the method entails.

[1] By this is meant the drop of fluid in which sowings of the fungal spores are made on the surface of the host plant. With Fungi of this type, this represents the usual procedure in artificial infection.

2. *Uniformity and Completeness of Germination.*

For this purpose, the factors of importance are—

(*a*) The spores must be uniformly distributed throughout the nutrient fluid;

(*b*) The quantity of spores per unit volume of nutrient should not exceed a certain limit;

(*c*) The film of nutrient on the plate should be of uniform depth throughout.

The details of the method, as given below, were only gradually evolved, and being based on numerous subsidiary experiments may be taken as representing the best set of conditions from the point of view on the one hand of effective and uniform germination, and on the other of economy of space and labour.

The method of dealing with 0·5 c.c. of centrifuged spores, which is about the amount obtainable from two, fairly good, 8 cm. diam., Petri dish cultures, may be stated. This quantity of spores is suspended in 50 c.c. of the nutrient fluid. The plates on which the spore suspension is sown are flat, circular, of 8 in. diameter. Each plate is supported on three wedge-shaped corks in the bottom of a large Petri dish, the atmosphere of the latter being kept moist by the usual devices. Each plate is accurately levelled by means of a spirit-level previous to sowing, this being effected by manipulation of the supporting corks. Immediately before each plate is sown, the spore suspension must be agitated. This can readily be effected by blowing in air through the pipette which is employed for measuring out the allowance for each plate. The spore suspension is now spread over the plates up to $\frac{1}{4}$ in. from the margin at the rate of 5 c.c. to each, spreading being most readily effected by means of the finger. With practice it is quite easy to sow as many as 20 plates in half an hour. The spores are now left to germinate at the ordinary laboratory temperature (about 20° C.).

With the plates employed and with the given suspension density of spores (0·1 c.c. spores to 10 c.c. nutrient) it has been found that any reduction from the amount given above (viz. 5 c.c.) for each plate has resulted in a diminution of yield together with increased tendency to lack of uniformity in germination. Any reduction in the amount of liquid should be accompanied by a corresponding reduction in the density of suspension.

In the earlier stages of this investigation, the nutrient fluid employed was a commercial grape preparation (*Welch's Grape Juice*), and for purposes of the manufacture of spore material this medium proved quite satisfactory. This preparation, however, when laid in the form of drops on the surface of plant tissues (leaves, &c.) has a very strong plasmolysing and killing effect; and in view of the fact that one object of the research was to

institute a comparison between the action of the extract obtainable from the germinating fungus and that of the fungus itself upon the living tissue, this feature was very objectionable, and an effort was therefore made to find a substitute. This was found in Turnip extract, which has proved to possess many advantages. Besides considerations of cheapness and availability, it is superior to 'Grape Juice' in its much slighter plasmolytic effects while equalling it in the quantity, and more than equalling it in the quality, of fungal material which it furnishes. It also possesses the considerable advantages that it is only slightly coloured, and its stock solutions can withstand repeated heatings without deterioration, whereas 'Grape Juice' suffers a definite loss by precipitation at each sterilization. This turnip medium is prepared as strong as possible—that is, the turnips (white) are autoclaved without addition of water, and the juice subsequently extracted under pressure. The most suitable medium is derivable from solid half-grown turnips, that from old vacuolated ones being considerably weaker and affording a smaller yield of germinated spore material.

The germination phenomena need not be described in detail. After four hours, commencement of germination can be seen in a small number of spores; after eight hours, germination is very general, but is variable in amount, the germ tube ranging from a mere papilla to a tube of four spore-lengths; at the end of the period allotted, apart from occasional spores which have remained clumped together and which may show various stages of arrested development, germination is very generally well advanced. The young hyphae, by reason of the comparatively thick rate of sowing adopted, are all closely intertwined and their individual appearances can only be readily seen by tearing out a small portion of the 'weft'. The average length of hypha depends naturally on the strength of the particular turnip extract employed, but it may be taken as approximating to 20–40 spore lengths. While each spore normally puts out a single germ tube, bipolar germination is frequent, in which case the length of each tube is less than in the normal case. Side branches of first order are not at all uncommon.

At the conclusion of the period of germination the plates thus present a continuous weft of interlocking hyphae, and the whole film may be removed as such, much in the same way as a gelatine film may be removed from a photographic plate after immersion in warm water. The fungous film offers usually considerable resistance to removal, being firmly attached to the plate, a phenomenon which finds its explanation in the very general development of incipient attachment organs. This is readily verified microscopically, a large proportion of the hyphae showing at their tips the flattened slightly swollen appearance characteristic of the early stages of these organs. The formation of these attachment organs is very strongly marked in sowings in Turnip extract, more so than for instance in sowings in 'Grape Juice', and it is at any rate plausible that the superior quality of

the material obtainable by using the former liquid is in some way related to this marked development of attachment organs.

In practice the removal of the film from the plate is most readily effected by means of a glass slide. The spore material is now thrown on to a muslin cloth, the edges of which are wired to a tripod in such a way as to form a bag, and the whole mass is subjected to vigorous washing, with stirring, under the tap. Any ungerminated spores, minor débris as well as the nutrient medium, are by this means washed away, while the weft-like nature of the mass of germinated spores prevents their passing through. This washing is continued for ten to fifteen minutes, and is followed by three successive washings in a large quantity (half a litre) of distilled water. The spore material is now strained as far as possible, spread evenly over a glass plate, and dried over calcium chloride *in vacuo*. The fungus material when dry is scraped off and ground in a mortar with clean, dry quartz sand. Throughout this work equal weights of fungus and sand have been used. If perfectly dry—i. e. immediately after removal from the desiccator after overnight exposure to the calcium chloride—the fungal skin is brittle and lends itself quite readily to grinding. When kept for some time in the ordinary laboratory atmosphere, being hygroscopic, it gains slightly in weight and in this state is tough and difficult to reduce to powder. In practice an attempt was made to make the grinding of successive lots as far as possible uniform in degree, this being done by the use of a system in grinding. The ground powder is a uniform grey, and the degree of grinding can be estimated roughly by the change in colour from the black of the unground to the light grey of the well-ground spores. Grinding has throughout been performed by hand, though some sort of mechanical apparatus could no doubt be readily fitted up. In all cases the amount of grinding has not been stinted, and examinations of a little of the wetted powder which have been made from time to time under the microscope have shown that only occasional spores escape destruction; recognizable traces of hypha are also rare.

As the materials of different days' growth vary to a slight extent in the activity of the extract to which they gave rise, the practice has been to collect a considerable quantity of material, which is then intimately mixed up before being used for experimental purposes. This method is justifiable on the ground that, as far as can be seen, the dry powder preserves its activity undiminished for a very considerable time. Thus in one instance a certain tube of material appeared to possess undiminished activity after a two months' interval.[1]

[1] This is in accordance with the behaviour of other similar substances in the dry form. In the present case an exact proof is impossible on account of the difficulty of obtaining identical substrata at different times. The above conclusion is based upon the general effectiveness of the material upon a variety of substrata.

The spore material is extracted by suspension in distilled water, care being taken to keep the débris in suspension by shaking at intervals. By subsidiary experiments (using the quantitative method to be described below) it was shown that by increasing the amount of powder suspended in a given volume of water, the activity of the resultant extract increased, but only up to a certain point, further increases in the proportion of powder giving no appreciable increase in the activity of the extract. This limiting activity of extract is obtained when extraction is made in the proportion of 0·2 gr. powder (= 0·1 gr. fungus + 0·1 gr. sand) to 3–4 c.c. water. Thus the difference between the activity of, say, a 0·2 gr. in 2 c.c. and a 0·2 gr. in 3 c.c. suspension is, with powder of normal quality, scarcely demonstrable. In view of these considerations, the spore powder has been extracted throughout in the proportion of 0·2 gr. to 3 c.c. water, the full activity for the powder being thus obtained.

In view of the statement of Michaelis (Abderhalden's Handbuch d. biochem. Arbeitsmethoden III, i, p. 13) that in such cases, where the enzyme or similar substance is contained on the surface of an insoluble powder, an extraction of twenty-four hours' duration is required in order to ensure uniformity of extract in different experiments, a series of experiments was set up to determine what time of extraction is necessary in order that the extract may reach its limiting strength. Extractions were made for periods of $\frac{1}{4}$, $\frac{1}{2}$, $\frac{3}{4}$, and 1 hour, the process being stopped at the end of each period by centrifuging off the débris. These extracts were compared by the quantitative method to be described later. In the case of the $\frac{1}{4}$-hour extract only was there any indication that the full strength had not been reached. The three other extracts were of equal strength within the limits of experimental error. Throughout this work, one hour's extraction has been allowed, this time being chosen as representing safety as well as offering conveniences for the carrying out of the other experimental details involved. The liquid is finally cleared by centrifuging for three minutes at the highest speed available (3,000 revs. per minute), decanted, and again centrifuged and decanted. The liquid thus obtained is the crude extract which has been employed in the present investigation. It possesses a pale straw-colour, is opalescent, and has a characteristic 'mouldy' smell.

Though the experimental method as sketched above seems somewhat laborious, it is by no means unduly so, and it is quite practicable to prepare in this way quantities of material comparable to those which are usually employed in enzymic studies. The following figures will suffice to show how the method works out, representing as they do a fair average:

From two Petri dishes of 8 cm. diameter are obtained 0·5 c.c. spores (wet volume); the latter are sown in 50 c.c. Turnip extract on 10 plates. The weight of germinated material when dried = 0·7 to 0·8 gr. Thus the powder obtained = 1·4 to 1·6 gr. For the preparation of standard extract,

this quantity of material is suspended in 21 to 24 c.c. water, furnishing after centrifuging 18 to 20 c.c. of extract. As the result of 26 days' preparation of material, a maximum of 10 plates being employed, a quantity of 35 gr. of powder was collected. This represents a quantity of about 420 c.c. of standard extract. Again, as the result of 10 days' preparation, about 25 plates being sown daily, a stock of upwards of 60 gr. of powder was gathered, representing about 750 c.c. of extract. It is thus plain that the experimental treatment of this subject, according to the routine here adopted, offers no greater difficulties from the point of view of the availability of material than are met with in enzymic studies generally.

In carrying out the above-described routine, no special precautions against bacterial contamination have been found necessary. Bacterial action would be considered most likely to show itself on the germination plates at the end of the germination period. No doubt bacteria do occur, though several examinations with the microscope have failed to demonstrate their presence. This state of affairs is probably to be set down partly to the acidity of the turnip medium, and partly to the vigorous development of the fungus spores. In any case a limited bacterial development is of no importance, as both bacteria and bacterial products are washed away in the further treatment of the fungal material.

D. Quantitative Method of studying Action of Fungal Extract.

The basis of the method is the capacity of the fungal extract to destroy the coherence of any susceptible tissue which is placed in it for a sufficiently long time. The method is as follows:

From a tuber or other fleshy organ, an axial cylinder of about 1–1¼ cm. diameter is cut by means of a cork-borer; this is cut across in the middle, and from the surfaces so exposed transverse sections of ½ mm. thickness are cut by means of a hand microtome.[1] The discs are now freed from contained air by injection under the pump with water. After thorough washing in distilled water, they are placed in the extract and the time noted that is required to bring about 'loss of coherence'. For purposes of the present quantitative test, *coherence is said to be lost, when the discs as tested by hand offer no perceptible resistance to a pulling stress.*[2] The time from commencement of action to 'loss of coherence' gives a measure (inverse) of the activity of the extract.

[1] Leitz, Wetzlar.

[2] This stage does not represent the end-point of the macerating action even in its macroscopical aspects. The discs later become so fragile that it is impossible to handle them without rupture, though they do not actually fall asunder of their own accord. This stage, however, is difficult to determine, as there is no means of regulating or measuring the small strains which bring about rupture.

The limitations of this method, and the accuracy of which it is capable, are discussed under the following five headings:

(i) *Nature of tissue employed.* It is obvious that a tissue suitable for this purpose is one which in its fresh state possesses a marked degree of coherence. Such a tissue, as for instance that of the pear or even of many varieties of apple, is of little value; on the other hand, turnip and potato tissues are eminently adapted to the present purpose, and these have accordingly been generally employed.

The ideal tissue is one which allows of discs being cut which are uniform in quality throughout. Ordinary leaf tissue cannot therefore be employed, and in practice we are confined to the use of fleshy tissues. Even with these it is in general impossible to prepare discs which are quite uniform throughout. Thus even in the centre of the potato small vascular strands occur which produce local lack of uniformity. In cases where these are abundant, the tissue should be rejected, and in all cases care must be taken that these small vascular strands do not run in the plane of the section and more especially in the direction in which coherence is tested. A tissue suitable for the present purpose may therefore be defined as one furnishing discs which on microscopical examination are seen to consist of a ground-mass of uniform parenchyma cells with a limited number of islands of vascular (more resistant) tissue.

(ii) *Accuracy in thickness of the sections employed.* The degree of accuracy obtainable in the disc-cutting process depends to a considerable extent upon the nature and physiological condition of the particular tissue. It also depends upon the thinness of the discs cut, there being a limit of thinness beyond which the microtome ceases to perform satisfactorily in this respect. Care must be taken that the cylinder of tissue is sufficiently rigid not to yield to the knife. This consideration requires that the cylinder, in addition to being of a certain stoutness, should be quite turgid, a condition which is readily obtained by injection with water previous to cutting. In the case of potato tissue, the upper limit of variation in thickness of the microtomed discs was determined as 4 per cent.[1]

(iii) *The determination of the 'end-point' of reaction.* It must be conceded that the arbitrarily chosen end-point does not represent any definite stage of the reaction. It is simply that stage at which

[1] This was measured by cutting a series of sections, which were then washed, rapidly dried between filter-papers, and weighed. The following were the figures obtained:

Max. weighing	0·088 gr.
Min. ,,	0·085 gr.
Average weight of sixteen discs	0·0867 gr.
Max. variation from mean	0·0017 gr.
Max. variation among the individual readings	0·003 gr.

This variation of 0·003 gr. in 0·0867 gr. is approximately 4 per cent.

coherence has been so far reduced as to be imperceptible when tested by the hand—that is, when the disc is pulled from opposite sides it separates without perceptible resistance. The exact position of this point in the series of changes in the tissue brought about by the extract obviously depends on the 'stimulus threshold' for resistance to pull of the particular observer. This limiting value varies with the same observer from time to time, and thus a varying personal equation is introduced. In cases where two extracts of approximately equal strength are being tested, it is obvious that if the determinations are made within a short time of each other, the personal variation is small. When two extracts of widely different activities are being compared, it may happen that the end-points are reached at widely different times, in which case the personal factor may be different in the two determinations. It is, however, plain that in such a case the necessity for accurately determining the end-point is diminished.

The accuracy of determination of the end-point obtainable in practice may be gauged from the following figures. In the case of an experiment which would be set down as finished in fifty minutes, it is quite possible to convince oneself that a definite degree of coherence is perceptible after forty-five minutes; in other words, it is quite feasible in an action lasting fifty minutes to determine the end-point to within five minutes. This gives an error limit of about 10 per cent. Furthermore, when discs from the same region of an approximately uniform tissue are tested with the same extract, their end-points are found to show agreement to within 10 per cent. This figure then represents roughly the limit of variation which ceases to be significant. In the following it will be seen that the differences of activity noted are as a rule much greater than this, and no conclusions are drawn from observed differences in activity in which it was not perfectly certain that the differences observed lay far beyond the limits of this observational error of 10 per cent.

(iv) *The varying nature of the actual substrate.* There is wide variation in sensitiveness of the different potatoes, turnips, &c., employed. Thus with the same extract a variation of 100 per cent. has been observed in different potatoes. The same has been shown to apply to turnips. This consideration renders the comparison of results of different series of experiments difficult, and in practice this was carried out by comparing each with a standard. This is furnished by the standard extract above described, a common stock of spore material being kept for purposes of this day-by-day comparison. When, however, the comparison of two sets of experiments was considered to be of critical importance, it was always possible to carry out the observations side by side on the same substrate.[1]

[1] It might be thought feasible to cut from the same potato a large number of discs, which could then be kept in presence of an antiseptic and used for standardizing purposes. This method

(v) *The stability of the extract.* The extract is not a stable solution, but loses its activity with lapse of time. After twenty-four hours in presence of chloroform, it has lost slightly in activity; after a week its activity is very small. The effect of this deactivation with time is to prolong the slow reactions unduly. This error can of course be corrected by renewal of the liquid from time to time. In the average experiment, lasting less than six hours, this procedure is not necessary, and the error introduced is not appreciable.

Other Quantitative Methods. A variety of other methods has been tried from the point of view of a quantitative treatment of the subject. As these have been comparative failures, it is only proposed to mention them briefly.

A substrate was prepared for the enzyme in the following manner. Turnip tissue was pulped, freed by prolonged washing from soluble materials, dried and ground to a powder. This may be considered to be in the main a cellulose powder. Water suspensions of this powder were added to the extract, and the action was estimated after a time by Fehling's solution. This experiment was controlled by a similar one in which deactivated (see later) fungus extract was employed. The conclusion reached was that while the figures lay in the right direction, they were far too small to encourage the hope that the method might be serviceable. Quite apart from being very laborious, the method appeared infinitely inferior to the one adopted. The same remarks apply to a number of experiments in which an attempt was made to use as substrate a 'calcium pectate' extract of turnip tissue, prepared according to the method described by Behrens (l. c.).

In another series of experiments the killing action of the extract was followed by measuring the rate of escape of certain constituents of the tissue. Experiments along these lines were tried with tissues of turnip, onion, and beet. In the first two of these the action was estimated by Fehling's solution, in the last it was measured colorimetrically. The conclusion reached was that this represented a possible method which, given a suitable tissue, might furnish results of value. It did not appear, however, sufficiently promising to warrant its continuation at the time.

An extensive investigation of plant pectins is at present being carried out in this laboratory by Dr. Schryver. It is hoped that on the basis of his results it may be possible to elaborate a suitable chemical method for standardizing fungal extracts as regards their capacity to dissolve the cell-wall.

is, however, quite unsafe, as there is no guarantee that the discs remain unaltered when so preserved. In point of fact, observations have shown that alterations do take place. Thus, if a number of discs from the same region of the same potato are preserved in different antiseptics, they are found after a week's interval to show widely different degrees of sensitiveness to the action of the fungal extract. Variations amounting to as much as 400 per cent. have in this way been produced. A more striking example of the same phenomenon is given later (p. 344). The above serves to illustrate the dangers attendant upon this method of standardizing extracts.

E. Action of Extract on Tissues.

(*a*) *General Account.*

The action of the extract is of a twofold nature:

1. Solution of certain constituents of the cell-wall, resulting in loss of coherence of the tissue.

2. Death of the cells themselves.

These two aspects of the phenomenon will in the meantime be referred to respectively as the 'macerating' and 'lethal' actions of the extract.

Fleshy tissues were prepared in the way described in the preceding section. The following tissues were tested and found to be readily acted on: Tubers of *Potato*, roots of *Turnip*, *Beet*, *Radish*; fruit tissue of *Apple*, *Cucumber*; pith of stem of *Senecio articulata*. With extract of normal strength, potato discs of $\frac{1}{2}$ mm. thickness are very usually disintegrated in twenty to thirty minutes, though, as has been stated, there may be considerable individual variation. Discs of white or yellow Turnip require as a rule a similar time, while the harder tissue of Swedes is more slowly acted upon. The above list is no doubt capable of wide extension, and it is indeed highly probable that the fleshy parenchymatous tissue of fruits, tubers, &c., is very generally susceptible to the action of this fungus extract.

Tissues of leaves, petals, &c., were treated in a variety of ways. Discs of these were submerged in the active extract, so that action proceeded from the margin (the cuticle presenting an impenetrable obstacle to the diffusion of the extract, as will be shown in a subsequent paper), or the extract was injected into the tissue by means of the air-pump. In a series of experiments the extract was injected into the tissue by means of a hypodermic syringe. The advantage of this method is that the tissues are maintained in a more normal condition during the course of the action than when they are submerged in liquid.

When the discs are merely submerged in the extract the action may be comparatively slow, being to a large extent limited by the rate with which the leaf disc becomes injected. This injection process, which also takes place when the discs are immersed in water, is somewhat obscure in principle;[1] in particular the fate of the air of the intercellular spaces is not quite clear. Injection proceeds chiefly along the line of the vascular bundles, especially from the proximal end. The course of the action is also influenced by the mechanical properties of the leaf discs. Thus, in the case of tulip petal discs, the disintegration of the more sensitive central tissue

[1] This phenomenon is probably related to that of the ascent of water in shoots which are kept in a saturated atmosphere. The injection would thus be due to an active pumping action on the part of the cells of the leaf; cf. Dixon, Proc. Roy. Ir. Acad., vol. iv, 1896–8, p. 627.

along the cut surface results in the rolling apart of the upper and lower halves, so that the extract rapidly gains access to all parts of the disc; in rose petal discs the same effect is produced by the rolling back of the lower cuticle.

When the active extract is injected into the tissue, the action may be very rapid. The most marked effects are seen in the case of floral structures, in which injection of the extract produces rotting and death within half an hour.[1] This has been found to be true of upwards of thirty species investigated, whence it would appear that the extract is destructive to floral structures in general. In the case of foliage leaves, the rate of action is as a rule much slower, but is nevertheless strongly marked in many cases—leaves of succulents, of *Vicia Faba*, *Viola*, *Petunia*, *Lactuca*, *Begonia*, &c. In the case of leaves of a hard woody nature it has not been shown in any case that the extract has any action. Thus leaves of *Aucuba*, pitchers of *Nepenthes*, have afforded negative results.

When tissues of lower plants were investigated in this respect, unexpected results were obtained. Nordhausen (l. c.) records certain experiments in which moss leaves showed themselves very sensitive to the action of *Botrytis*, and it was in view of this statement, as also on account of their softness, that tissues of Bryophytes were expected to succumb rapidly to the action of the extract. Quite the opposite result was obtained, and in fact it is not too much to say that these forms show complete resistance to the action of the fungal extract. The following have been investigated:

Thallus of *Pellia*, *Fegatella*; leaves of *Plagiochila*, *Funaria*, and *Mnium*.

In no case whatever was any definite alteration demonstrated, even after several days' action of the extract, the latter being renewed from time to time.[2]

In view of this startling discrepancy, experiments were set up to see if any confirmation could be obtained of Nordhausen's statements as to the action of the fungus itself. For this purpose small clumps of the above-mentioned plants were sprayed with a turnip extract suspension of *Botrytis* spores, which were then allowed to germinate. In the course of a few days the clumps were quite infested with the fungus, becoming in fact totally covered and hidden by the mass of mycelium. Even after this drastic treatment, the plants, both mosses and hepatics, were found to be only very slightly affected, appearing slightly limp and unhealthy, but showing no

[1] In the case of the soft petals of *Gloxinia*, *Achimenes*, *Tradescantia*, *Saintpaulia*, &c., the effect of the extract is apparent within five minutes of the time of injection. Further action of the extract leads to almost complete solution of these petals.

[2] In the case of the moss leaves examined, groups of cells here and there were seen to be discoloured, but in no case was anything like a general action of the extract shown. Similar and, as far as could be judged, equal discolorations were seen in the control moss leaves which had been kept in water.

rotting nor any invasion by the fungus. The slight effects observed were set down to bacterial contamination, the cultures having become by this time very foul. In another experiment, pieces of *Pellia* thallus, leaves of *Mnium* and *Plagiochila* as well as portions of various higher plants (leaves of *Geranium*, *Dahlia*, petals of ditto) were placed on a plate of germinating spores, set up as previously described, control pieces being placed on a similar plate from which the spores were absent. In the course of twenty-four hours the *Geranium* and *Dahlia* tissues were largely invaded and decomposed; after forty-eight hours they were entirely rotten, while the controls were still unchanged. On the other hand, up to the end of the experiment, which lasted for four days, the moss and hepatic tissues appeared quite unaffected, although they had been completely overrun by the fungus.

The conclusion from these experiments, viz. that mosses and hepatics show a considerable degree of resistance to the attack of *Botrytis*, seems more in accordance with experience than the results of Nordhausen. The habitat of these plants is one which would be calculated to encourage the growth of the fungus. If they are as highly susceptible to attack by the fungus as Nordhausen states, it is surprising that no natural occurrence of the latter on these plants has been described. Apart from the experiments of Nordhausen, there appears to be no case of infection of mosses by *Botrytis* recorded.[1]

Among Algae, the action of *Botrytis* extract was tested on filaments of *Spirogyra*. From the two tests made in this connexion it appeared doubtful whether any action occurred. If any, it was certainly very slight. Here by way of contrast it may be mentioned that experiments with the filamentous staminal hairs of *Tradescantia virginica* gave a strong positive result, the filaments rapidly breaking up into the individual cells, from which the coloured contents soon became discharged.

Viewed generally, the macerating action of the extract shows itself in the tissue losing completely its coherence, subsequently breaking down to form a mush, and in extreme cases passing almost completely into solution. With regard to the lethal action, the criteria must to a large extent be chosen to suit the particular circumstances. In the case of such tissues as turnip or cucumber, the lethal action of the extract can be demonstrated by failure of the cells after a time to show plasmolysis in hypertonic solutions.

[1] The contradiction is in certain respects not absolute. Nordhausen took leaves of *Mnium*, laid them on the surface of a nutrient jelly, and sprayed *Botrytis* spores over the upper surface. The spores grew down into the cells of the leaf, being attracted chemotropically by the nutrient passing up from the underlying layer of jelly. The moss cells were killed, their contents discoloured, and the cell cavities finally became filled with a mass of hyphae. On the last point it is difficult to see how any mistake could have arisen, and it may be that the absence of a special chemotropic factor in the experiments of the present paper may have been responsible for failure to produce infection.

In coloured cells, death is as a rule shown by escape of the coloured substance. This substance may remain as such and impart its colour to the fungal extract (e. g. Beet), but more generally it disappears from view altogether (e. g. Rose, *Viola*, and many other petals), though its presence may be demonstrated by the addition of a suitable reagent (e. g. an acid in the case of the red pigment of Rose petals). In other cases the incidence of death is shown by a new development of colour, due to autolysis, i. e. to actions taking place which were held in abeyance during life. Such is the well-known black coloration produced after death in the leaf of the Broad Bean.

These post-mortem phenomena as witnessed in the various tissues experimented upon do not call for detailed description here. It is important, however, to state that the post-mortem changes brought about by the fungal extract, with one exception which is only apparent,[1] were identical with those induced by the action of the fungus itself. Again in all cases, where a distinct parasitism of the fungus on a particular host could be established, it was found that the tissues of the latter were acted upon in a similar way by the fungal extract. These considerations—viz. similarity of 'range', and similarity of effects produced—justify the conclusion that the standard extract of the present investigation is a true representation in essentials of the active principle of the fungus.[2] It is proposed, therefore, on the basis of these results, to put forward the following as a working hypothesis—*that all the macerating and lethal effects of the fungus can be explained on the basis of the properties of the standard fungal extract.*

[1] In the case of the leaf of *Vicia Faba*, it was found that the discs, when injected and submerged in a quantity of fungal extract, did not show the characteristic blackening produced by the fungus itself, but remained for a considerable time quite green, fading later into yellow. There was no doubt as to the discs being killed, for within a few hours from the commencement of the experiment they became quite limp and rotten, and the cells were no longer capable of plasmolysis. Moreover, no blackening effect appeared if these discs were subsequently placed in chloroform vapour. It was by reason of this discrepancy that the hypodermic syringe method was first resorted to, when it was found that leaves injected with extract according to this method gave the normal blackening. The explanation of these appearances is fairly simple. The black pigment is formed as the result of an oxidase reaction, in which the source of oxygen is the free oxygen of the atmosphere. The latter is available in the case where the leaf is injected by means of the hypodermic syringe; not so when it is injected and immersed in some depth of liquid. In the latter case, at the time and place of killing, one of the factors essential for the development of the black colour is to a large extent wanting; the two remaining factors concerned (enzyme and oxidizable substance) diffuse out into the liquid, where they gradually meet the atmospheric oxygen, so that a development of the black pigment takes place slowly in the liquid. This last had, in fact, been noticed long before the apparent discrepancy was understood.

[2] In Smith's experiments, treatment of lettuce leaf with oxalic acid produced a bleaching effect, in contrast with the browning effect produced by the fungus and fungal extract. These results have been confirmed, and in the opinion of the present writer this disparity in post-mortem effects constitutes a strong objection to the view that oxalic acid is the toxic principle of the fungal extract and of the fungus.

(*b*) *Detailed Account of certain Cases.*

As has been stated, the action of the fungal extract on a tissue is of two kinds, a macerating and a lethal. The following account refers to an examination of the course of the action which was carried out with the object of establishing the time relationships of the two manifestations.[1] The criterion of maceration was as usual diminution of coherence. The criterion of lethal activity was the failure of hypertonic solutions to cause plasmolysis.

It will be noted that of the two phenomena to be studied, the one entails microscopic, the other (in the main) macroscopic observation. In order to make correlation possible it is obvious that homogeneity of tissue is all-important. We are thus limited to fleshy parenchymatous tissue. Even here the ideal tissue is not obtainable. Thus in turnip tissue homogeneity is disturbed by the presence of small vascular bundles, the small cells in the neighbourhood of which are more slowly acted upon than the larger cells of the ground-mass. In other cases—e. g. in the stem (pith) of *Senecio articulata*—there is a gradation from the large, more sensitive cells in the centre to smaller, more slowly reacting cells towards the periphery. The difficulty, however, is not so great as might appear. The coherence of a disc of any particular tissue is no greater than that of its weakest part, *and we have accordingly to compare the progress of the macerating action, as shown by the loss of coherence, with the plasmolytic features of the more sensitive portions.* Thus in turnip tissue we correlate the condition with respect to coherence with the condition as regards plasmolysis of the large cells of the ground-mass ; and similarly for the case of the large central cells of the pith of *Senecio articulata.*

The tissues which have been found useful in this connexion are as follows : [2]

Turnip (*white*): nearly full grown; must not be old and vacuolated; discs cut from an axial cylinder in the neighbourhood of the centre.

Swede: possesses the great advantage that uniform discs of $\frac{1}{5}$ mm. thickness can be cut.

Cucumber: discs from axial cylinder in the region of the basal contracted portion.

[1] Van Hall (l. c., p. 135), from microscopic examination of the tissue lying between the sound and the decomposed tissue, states ' that it is sometimes possible to observe one or two layers of tissue, the cells of which still hang together, although, as can be seen from their contracted protoplasts, they are already dead '. From this he concluded that the cells are first killed and then isolated from each other.

[2] The potato, which has proved of great value in this investigation, is useless for the present purpose on account of the large amount of solid cell contents present, which makes a plasmolytic study impracticable.

Senecio (*Kleinia*) *articulata*: Transverse sections of stem, the sides being cut away so that the sections take the form of narrow strips including the central tissue of the stem.

Cotyledon rosacea: Transverse sections of leaf, prepared in the same way as the immediately preceding.

In all cases the tissue discs or strips were prepared according to the method for quantitative experiment; previous to use they were injected with water.

The following account of an experiment with *transverse axial strips of pith of Senecio articulata* will serve to illustrate the sequence of phenomena observed:

Strips placed in standard extract at 4.5 p.m. 4.20, strips coherent; ditto at 4.25 and 4.30. 4.35, coherence gone.

A strip at this stage is placed for three minutes in a 5 per cent. solution of KNO_3 in which a little eosin is dissolved; then washed in 5 per cent. solution of KNO_3. On microscopic examination, this strip shows as many fully plasmolysed cells in the central region as a strip which has not been exposed to the action of the extract. The cells of the strip exposed to the extract only differ in appearance from those of the latter inasmuch as the cell-walls do not appear so well defined and are more transparent. There is no marked swelling of the walls.

If a strip which has been treated with extract be pulled apart at this stage, it shows a characteristic appearance along the line of separation. Separation follows the line of the cell-walls, the cells on either side being left intact. When, on the other hand, a strip of tissue which has not been treated with extract is pulled apart at the centre, the line of separation passes as often as not across the cells so that the tissue at the margin possesses a ragged appearance. This phenomenon is taken as indicative of the action of the extract upon the middle lamella, and it shows that the latter is in an advanced state of solution at a time when the cells are still alive and when the remaining portions of the cell-wall still possess their mechanical properties.

4.50—a strip on plasmolysis still shows a large proportion of live cells in the central region, quite close up to the line where the strip separates on being pulled. The tissue is now very difficult to handle without producing damage. Thus when a cover-glass is laid on a portion of the central tissue, the latter becomes a disorganized mush. The collapsed cell-walls of this mush show a flaky or layered appearance, indicating solution of certain cell-wall constituents, a process which results in the cell-wall losing its mechanical properties.

5.5 and 5.20—a fair proportion of central cells still plasmolysable.

5.50—central region now shows only occasional living cells, the protoplasts of which are reduced by plasmolysis to very small globules; the

tissue is now to a large extent a mush even without the disturbance incidental to the addition of a cover-glass.

Thus in the present case, while the tissue coherence becomes reduced to a negligible quantity in thirty minutes, the cells themselves in the most sensitive region are all alive at this stage, and a certain proportion are still alive after seventy-five minutes.

The following represents a summary of similar experiments in this connexion:

Turnip (white) discs: coherence gone in 15′; considerable proportion of plasmolysable cells after 20′: this proportion much reduced after 30′.

Cucumber discs (weak extract employed): coherence gone in 25′: large proportion of plasmolysable cells after 45′: this proportion much reduced after 60′; only an occasional plasmolysable cell after 90′.

Turnip (white) discs (weakened extract): coherence gone in 90′; proportion of plasmolysable cells undiminished after 120′; proportion distinctly decreased after 135′, but a fair percentage of plasmolysable cells still remains after 150′.

Turnip (Swede) discs of ½ mm. thickness (weakened extract): coherence gone in 40′; proportion of plasmolysable cells undiminished at this stage, also at 50′ and 55′; slight reduction after 60′, but still considerable proportion of live cells after 70′; number of plasmolysable cells small after 90′.

As comparable with the preceding may be cited certain experiments with the staminal hairs of *Tradescantia virginica*. These are rapidly disintegrated by the fungal extract, so that after a time it is easy by gentle shaking to cause them to break up into their individual cells; if treated at this stage with 5 per cent. KNO_3 the cells are found to be almost universally plasmolysable.

On the other hand, cases were found where this time separation of macerating and lethal effects could not be effected. This was the case with sections from the fleshy leaf of *Cotyledon arborea*, and also to a less extent with sections from old white Turnip. Here it was found that at the stage where coherence was lost considerable disintegration of the cell-walls could be demonstrated and a large proportion of the cells were no longer plasmolysable.

Summing up, the action of the extract on living tissue is as follows:

The first noticeable change is the solution of the middle lamella, so that the tissue loses its coherence. At the stage which is termed 'coherence gone', this action has progressed so far that the middle lamella has lost its mechanical properties as a solid layer 'cementing' the cells of the tissue together. As a consequence the tissue readily falls apart along the line of the middle lamella. At this stage, however, the remaining layers of the cell-wall possess in some degree their original mechanical properties, and the cells themselves are quite alive. Very soon the remainder of the cell-wall is disintegrated, breaking down into what appear to be flakes or

lamellae, and the whole structure becomes very fragile. This feature becomes more and more pronounced, and in course of time the tissue falls into a 'mush'. In no case has complete solution of the cell-wall been seen. Death of the cells takes place some time after fragmentation of the cell-walls is definitely established; the latter process, in the majority of cases examined, is not in evidence at a time when the tissue has lost all coherence as a result of the solution of the middle lamella.

F. Examination of certain Physical Relationships of Extract.

This investigation was undertaken in the first instance with the object of trying to effect a separation between the 'macerating' and 'lethal' principles of the extract. As the research proceeded it was, however, found expedient to develop it on broader lines. This examination in certain parts is not yet completed.

Relation to Heating.

The activity of the extract, both as regards macerating and lethal effects, is totally destroyed by a sufficient degree of heating.

Below 55° C. deactivation is comparatively slow; above this temperature it becomes very rapid, and at 65° it is as near as may be instantaneous. To study the effect of heating between the limits of 50° and 65°, the following method of heating was adopted by way of standard:

A small tube containing 2 c.c. of extract was dipped into a beaker of water which was kept at a temperature 5° higher than the temperature desired. A thermometer served the double purpose of stirring the contents of the tube and recording the rise of temperature. The tube containing the extract was taken out immediately the required temperature was reached. This heating process, for temperatures between 50° and 65°, occupies approximately half a minute.

The following table shows the rapid nature of heat deactivation when a temperature of about 55° is reached. The heating process was carried out in each case as described above.

'Macerating activity' = reciprocal of time required to cause loss of coherence, that of unheated extract being taken as unity.

Extracts tested on Turnip Discs.

Treatment.	*Macerating activity.*
Heated to 50°	1
,, 53°	1—
,, 55°	[illegible]
,, 58°	[illegible]
,, [illegible]	[illegible]
,, 63°	[illegible]
,, 65°	0

At and below 50° deactivation is relatively slow, as is shown by the following figures:

Treatment.	*Macerating activity.*
Heated at 50° for 15 min.	$\frac{7}{18}$
„ 50° „ 45 min.	$\frac{1}{2}$
„ 45° „ 1 hour	$\frac{2}{3}$
„ 40° „ 1 hour	$\frac{8}{9}$
„ 35° „ 1 hour	1

In all of the above cases where deactivation of the macerating principle was partial, it was seen that no separation of macerating and lethal principles had been effected. The lethal effect was tested in the case of the stronger solutions by injections into bean leaves; the weaker (much deactivated) extracts were tested as to their killing action on the more sensitive petals of *Gloxinia*. In all such cases a lethal action could be demonstrated. With extracts which had been completely deactivated in respect of their macerating action by heating to 65°, the lethal action was also completely stopped. This point is illustrated by the following experiment:

Two discs of *Crocus* petal were injected under the air-pump, the one in unheated extract, the other in extract which had been heated to 65°. Both extracts had been previously cooled to nearly 0° C., and throughout the experiment were kept in an ice chest. The disc injected with unheated extract was completely disorganized in an hour: that in the other was alive and apparently unaltered after five days, at which time, as bacteria were now beginning to appear in numbers, the experiment was discontinued.

Throughout this work, extract which has been heated in the manner described to 65° has been used as the standard control.

It is noticeable that heat deactivation causes a certain amount of coagulation in the extract, which is seen to become more opalescent. This, however, has nothing to do with the phenomenon of deactivation, as the extract when purified in certain ways shows the same sensitiveness to heating, but without exhibiting any coagulation.

Relation to Mechanical Shaking.

The extract may be deactivated by mechanical agitation—e. g. by bubbling air through it or by shaking in a closed vessel. As it is proposed to publish the results of this investigation separately, no detailed description will be attempted here. The following figures illustrate the magnitude of the effect:

(Agitation produced by a stream of bubbles.)

Activity of extract maintained (but not shaken) at 35° for 1 hr. = 1.

„ „ „ shaken at 35° for $\frac{1}{2}$ hr. = $\frac{2}{5}$.

„ „ „ shaken at 35° for $\frac{3}{4}$ hr. = $\frac{1}{9}$.

As in the case of heat deactivation, it was found that no separation of macerating and lethal actions could be effected by this means.

Relation to Diffusion and Dialysis.

The experiments on this subject are not yet completed, and it is therefore proposed to give certain results only in *résumé*.

Comparative diffusions were carried out by means of a series of graded gelatine (5, 10, 15, 20 per cent.) membranes prepared after the method of Bechhold. From these experiments it appeared that the macerating principle possessed a coefficient of diffusion comparable with that of *dextrin* and greater than that of *diastase*. It is thus a colloid of intermediate type. Again, all the fungal extracts which have been purified by diffusion through gelatine membranes showed a normal lethal activity, and though it has not been possible as yet to perfect the experiments in this connexion, there is a strong presumption that if the diffusate possesses any macerating activity, it possesses likewise lethal activity—in other words, if there are two principles concerned, the diffusive capacity of the lethal principle is not less than that of the macerating one.

A complete dialysis of the macerating principle was effected by the use of a certain type of collodion thimble. In this case it was found that the macerating principle could be completely held back. On testing the dialysate, after removal by means of the air-pump of the volatile antiseptic, no trace of a killing action was shown. Subsequent control experiments with the same membranes showed that they were quite permeable to crystalloids such as cane sugar, ammonium oxalate, &c.

By this dialysis method a very convincing proof was obtained that soluble oxalates do not play any part whatever in the lethal activities of the extract. The dialysate from the collodion thimbles gave a precipitate with potassium oxalate solution, thus showing the presence of a substance (presumably a calcium salt) which precipitated oxalates. Control experiments showed that this substance was not derived from the membrane itself. It was accordingly present in solution in the continuous phase of the colloidal extract, so that the simultaneous presence of a soluble oxalate was quite excluded.[1]

[1] This dialysis experiment was in one case carried out with the following precautions: The collodion membranes were never allowed at any time to come into contact with tap-water. The germinated spores were subjected to prolonged washing in distilled water and dried *in vacuo* over sulphuric acid. The dried material was ground without sand, a control experiment having shown that no calcium was derivable from the mortar. With extract from this material it was also found that calcium could be demonstrated in the dialysate. Since these precautions for excluding contamination with traces of calcium were not usually adopted, it is safe to assume that the presence of a soluble calcium salt can be predicated of all the extracts that have at different times been employed. Whether the calcium salt is derived from the contents of the fungus cells or is strongly adsorbed on the walls from the liquid in which germination took place, it is impossible to say.

It appears, therefore, that by none of the methods above described was it possible in any way to obtain any separation of the macerating and lethal actions of the extract.

G. Relation of Activity of Extract to certain Chemical Substances.

Acidity of Extract.

The standard extract shows a weak acid reaction. With any of the usual indicators, the neutral point is not sharply defined, so that the acidity can only be measured approximately. With phenolphthalein, $\frac{n}{160}$ was established as upper limit of acidity; with neutral red the acidity was given as $\frac{n}{180}$ to $\frac{n}{200}$. During the process of neutralizing the latter indicator changes continuously from red to yellow. This would indicate that the acids present are of a very weak or of a polybasic nature.

Effect of varying the Acidity on the Activity of the Extract.

(i) *Diminution of Acidity.* As the acidity of the extract is diminished, the activity remains unaltered up to a certain point, when it falls sharply to zero. In alkaline solution the extract has no activity. The following table will serve to illustrate the sharp effect produced on neutralization of the extract:

Indicator in each case = 1 drop of $\frac{1}{10}$ per cent. Solution of Neutral Red.

20 drops of Alkali solution = 1 c.c.

No.	*Extract.*	*Indicator.*	*Acidity.*	*Time to decompose turnip disc.*
1.	5 c.c. Ext. + 6 drops water	red	$\frac{n}{200}$	18 min.
2.	5 c.c. Ext. + 4 „ „ + 2 drops $\frac{N}{10}$ NaOH	grading	$\frac{3n}{1000}$	18 min.
3.	5 c.c. Ext. + 3 „ „ + 3 „ „	continuously	$\frac{2n}{1000}$	20 min.
4.	5 c.c. Ext. + 2 „ „ + 4 „ „	into	$\frac{n}{1000}$	35 min.
5.	5 c.c. Ext. + 1 „ „ + 5 „ „	clear, permanent	0	100 min.
6.	5 c.c. Ext. + 0 „ „ + 6 „ „	yellow	$\frac{n}{1000}$ alk.	> 20 hrs.

In the case of (6) the disc remained coherent and quite alive (i.e. turgid) after 20 hours. In the remainder, death of the cells followed in the usual way. This shows that the lethal principle of the extract reacts as

sharply and at the same point to the addition of alkali as does the macerating principle.[1]

When the concentration of alkali is further increased, a macerating action again sets in. Control experiments show that this is due to the alkali itself. With potato tissue a macerating action sets in when the concentration of alkali approaches the value $\frac{n}{50}$: with tissue of white Turnip a concentration of $\frac{n}{100}$ alkali can be seen to produce a slow macerating effect. It is noteworthy that maceration by dilute alkali is also accompanied by death of the cells.

The active principle is inhibited but not destroyed by the alkali. On adding an appropriate amount of acid, the activity of the extract is restored, that is, apart from the retarding action of the salt thereby produced.

(ii) *Increase of Acidity.* In this connexion the following acids have been tested—*citric*, *malic*, *tartaric*, *sulphuric*, and *hydrochloric*. In the case of the two mineral acids and of the dilute concentrations of the organic acids, the required concentration of acid in the extract was obtained by adding a measured quantity (in drops) from a strong solution of appropriate strength. The slight degree of dilution of the principle of the extract which is thereby produced is known to be of no importance. The higher concentrations of the organic acids were obtained by evaporating solutions of the strength desired to dryness on the water bath, and making up to the original volume with standard extract.

In all cases the action of the acid on the activity of the extract is one of retardation. The retarding effect in relation to normality is approximately equal for all the acids up to a certain point $\left(\frac{n}{32} \text{ to } \frac{n}{64}\right)$; above this point the retarding action for the mineral acids increases very much more rapidly than that of the organic acids. In the case of the mineral acids, the macerating action of the acid appears at a fairly low concentration, so that above this point the action of the acid extract is not distinguishable from that of the acid itself. In the case of the organic acids macerating effects due to the acid appear at a much greater concentration, so that the retarding action of the acid upon the extract can be studied over a comparatively wider range.

[1] As the colour reaction is not sharp, it may well be that (6) represents the neutral point more closely than does (5). Furthermore, there is an escape of acid juice from the turnip disc, so that the acidity of the extract varies with time, especially in the neighbourhood of the neutral point. Thus the activity assigned to extract (5) in the preceding table is probably too high, or, more exactly, the activity of that extract increases with the gain in acidity due to the escape of acid sap from the turnip disc. It is therefore impossible to say whether the activity of the extract disappears when the neutral point is reached or whether deactivation is completed when the solution reacts alkaline.

The following tables illustrate the above statements:

(*a*) In dilute concentrations (up to $\frac{n}{64}$), there is considerable agreement among the acids employed: the retarding effect of each increases gradually with the concentration. The figures represent the time in minutes required to produce loss of coherence in potato discs.

Acid.	$\frac{n}{\infty}$	$\frac{n}{1024}$	$\frac{n}{512}$	$\frac{n}{256}$	$\frac{n}{128}$	$\frac{n}{64}$
Citric	20–25	20–25	25	30–35	35	35–40
Malic	20–25	20–25	25	25–30	35	35–40
Tartaric	20–25	20–25	25	30–35	35	35–40
Sulphuric	20–25	20–25	25	30–35	35–40	40
Hydrochloric	20–25	20–25	25–30	30–35	35–40	40

(*b*) In the case of sulphuric and hydrochloric acids, the retarding action increases very rapidly above a concentration of about $\frac{n}{32}$.

Acid.	$\frac{n}{\infty}$	$\frac{n}{128}$	$\frac{2n}{128}$	$\frac{3n}{128}$	$\frac{n}{32}$	$\frac{n}{17}$	$\frac{n}{12}$	$\frac{n}{9}$	$\frac{n}{7}$	$\frac{n}{6}$	$\frac{7n}{38}$
H_2SO_4	20–22	30–35	35	35	90–105	⊕	⊕	+	+	+	+

⊕ These discs and those of the acid controls do not lose coherence within 24 hours.

\+ These discs lose coherence, the more rapidly the higher the concentration of acid; equal effects are shown by the acid controls.

Hydrochloric acid shows very similar effects: thus a concentration of $\frac{n}{15}$ stops the macerating action of the extract.

(*c*) The retarding action of the organic acids above the concentration $\frac{n}{32}$ is much less than that of the mineral acids:

Acid.	$\frac{n}{\infty}$	$\frac{n}{160}$	$\frac{n}{40}$	$\frac{n}{10}$	$\frac{n}{5}$	$\frac{2n}{5}$	$\frac{3n}{5}$	$\frac{4n}{5}$	n
Citric	20	30	40–45	40–45	45	75–90	120–135	210	⊕
Malic	20	30	40–45	40–45	45	75	90–105	135–150	$>4<18$ hrs.
Tartaric	20	35	40–45	40–45	45	135–150	$>18<30$ hrs.	⊕	⊕

⊕ Discs do not lose coherence in 3 days.

In all these acids the retarding action is approximately equal and is not considerable, up to $n/5$; above this concentration it increases more rapidly. The specific retarding action is least in malic and greatest in tartaric acid.

Effect of Salts and other Substances on the Activity of the Extract.

Salts. Here, as in the case of acids, the action is one of retardation. The variation of the retarding effect with concentration was followed in detail in the case of potassium nitrate. The magnitude of the retarding effect will appear from the following figures, potato discs being employed.

	$\frac{m}{\infty}$	$\frac{m}{572}$	$\frac{m}{256}$	$\frac{m}{128}$	$\frac{m}{64}$	$\frac{m}{32}$	$\frac{m}{16}$	$\frac{m}{8}$	$\frac{m}{4}$	$\frac{m}{2}$
(1)	15	15	15	15+	25	40	75	180	$3\frac{1}{2}$–4 hrs.	10–20 hrs.
(2)	15	15	15	15+	30	45	90	–	–	–

The amount of agreement obtained in experiments of this sort may be judged from comparison of the results of the two experiments tabulated above. A similar investigation with respect to KCl gave retarding effects somewhat less than those of KNO_3.

A detailed investigation of a variety of salts was not attempted. A certain number were, however, tested in this respect in order to see if the effects were in any way general; and in particular a salt of calcium was included in the list with a view to determining if it exerts any special action upon the extract, seeing that calcium is known to be a constituent of the middle lamella. The figures are given in the table below. In the case of each salt solution employed, it was previously ascertained that it showed a neutral reaction (this precaution must be particularly exercised with solutions of calcium chloride, which are liable to contain free alkali).

Time for Standard Extract = 17–20′.

Salt.	$\frac{m}{64}$	$\frac{m}{16}$	$\frac{m}{4}$ *and* $\frac{m}{2}$
NaCl	60	165	×
KCl	20+	70	×
NH_4Cl	20+	135	×
Na-Ac	70	90	×
K_2Ox	50	×	×
Na_2SO_4	40	90	×
K_2SO_4	70	115	×
$MgSO_4$	×	×	×
$CaCl_2$	50	×	×

× Coherent after 3 hours; devoid of coherence after 20 hours.

A subsidiary experiment with the last three salts showed that in no case were the retarding effects due to alteration of the tissue by the salts employed.

As expected, different salts show marked variation in their retarding actions. It is not proposed to draw any general conclusions from the above figures, as it has not been thought fit for the present to pursue this subject intensively. It appears, however, that no special action is to be ascribed to the calcium salt in this connexion. Also, the strong retarding effect of the magnesium sulphate is noteworthy.

Of other *crystalloidal neutral substances*, only two have been investigated—saccharose and glucose. No definite retarding effect has been demonstrated in the case of these.

Time for Original Extract = 30′.

	$\frac{m}{64}$	$\frac{m}{16}$	$\frac{m}{4}$	m
KNO_3	55	150	10 hrs.	
Saccharose	30	30	30–35	30–35
Glucose	30	30	30–35	30–35

While it would appear that the retarding action of a chemical substance is related to its capacity of ionization, it is still quite possible that some non-ionizable substances may exert a specific retarding or even an *anti*-action upon the principle of the fungal extract. In this connexion one may suggest such substances as tannins, alkaloids, latex constituents, &c. In any investigation of immunity from the biochemical side, experiment along these lines might prove to be of value. As, however, the specialization in relation to host is very slight in the case of the fungus under consideration, such a detailed investigation has not been attempted in the present case.

Traces of alcohol, chloroform, acetone, and formaldehyde do not, as far as can be seen, have any appreciable effect upon the macerating action of the extract.

Colloidal substances might be expected to influence the activity of the extract by disturbing the adsorptive equilibrium within the liquid. In this connexion only one colloid has been tested—viz. starch. The investigation of this point arose incidentally in the examination of the effects of dilution on the activity of the extract. An account of these experiments may conveniently be given here.

Three diluents were used: (1) water, (2) ½ per cent. starch solution, (3) extract deactivated by heating to 65°. The figures in the table represent the times required to produce loss of coherence in potato discs.

	Diluent.		
Concentration.	*Water.*	*½ % Starch Solution.*	*Deactivated Extract.*
1	20	20	20
½	25	25	25
¼	40	40	35
⅛	65	65	45

From this it appears that the starch has no measurable effect in the concentration employed. The greater activity of the extract diluted according to the third method is somewhat interesting. It would appear to be connected with the maintenance of the original acidity, the acidities of the extracts diluted with water or with starch solution being reduced very nearly to zero. (See p. 338, on effect of reduction of acidity on activity.)

From the fourth column of the above table we see that the activity of the extract is proportional to the square root of the concentration of active principle. In the neighbourhood of the full strength of the extract (i. e. with 'standard' extract) there is considerable divergence from this law. Here the decrease in activity on dilution is less than required by the law, or conversely the gain in activity with increase of concentration becomes negligible after a certain concentration is reached. This is in agreement with what was mentioned in dealing with the technique of extraction, where

it was stated that extracts of greater concentration than the standard did not appear to possess any increased activity. This asymptotic effect suggests that some other limiting factor comes into play when the higher concentrations are reached. Such a factor may be the rate of diffusion of the active principle into the tissue of the discs experimented with. The above considerations also show how it is that in certain cases a better comparison of the strengths of two extracts may be obtained by using the diluted in place of the extracts of full strength.

Influence of Plant Juices on Activity of Extract.

That plant juices retard the action of the fungal extract was known at an early stage of this investigation, and in fact this knowledge formed the starting-point of the experiments which have just been described. A series of experiments was now carried out to see if there is much difference among various plant juices in respect of the effect they produce upon the fungal extract; and more particularly, to see if any parallelism could be traced between the specific retarding effect of the plant extract and the relative immunity of the plant itself to the action of the fungus or of the fungal extract. It was known that a high degree of resistance to the action of the extract was shown by tissues of mosses and hepatics; and it was therefore of considerable interest to determine if this want of activity of the fungal extract was due to its deactivation by some specific anti-body capable of extraction from the resistant tissue.

The plant juices were prepared without dilution by squeezing through unsized filter cloth under high pressure. The crude turbid juice was cleared as far as possible by centrifuging before being used. The following table gives the figures obtained.

By 'concentration $\frac{1}{3\,6}$' is meant that in each 1 c.c. of the liquid to which the figure refers there were $\frac{1}{3\,6}$ c.c. of plant juice and $1-\frac{1}{3\,6}$ c.c. of fungal extract; and so on.

Column A = extract made by suspending 0·2 gr. *Botrytis* powder in 3 c.c. plant juice.

Plant.	*Concentration of Plant Juice.* 0	$\frac{1}{3\,6}$	$\frac{1}{1\,8}$	$\frac{1}{1\,2}$	$\frac{1}{5}$	$\frac{1}{2}$	A	*Remarks.*
Cotyledon rotundifolia leaf	1	$1-\frac{1}{3}$	$1-\frac{1}{2}$	$1-\frac{2}{3}$	$\frac{1}{4}$	$\frac{1}{1\,1}$	$\frac{1}{1\,0}$	Clear.
Bean leaf	1	1	1 (−)	1 (−)	Very variable.	0	0	Turbid.
Lemon fruit	1	$\frac{1}{2}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{1}{2}$	$\frac{1}{1\,0}-\frac{1}{4\,0}$	$<\frac{1}{4\,0}$	Clear. Acidity = 1·1 N.
Orange fruit	1	$\frac{1}{2}$	$\frac{1}{2}-\frac{2}{3}$	$\frac{2}{3}$	$\frac{1}{2}$	$\frac{1}{4}-\frac{1}{5}$	$\frac{1}{4}$	Clear. Acidity = 0·2 N.
Potato tuber	1	$1-\frac{2}{3}$	$1-\frac{2}{3}$	$\frac{2}{3}$	$\frac{2}{3}$	$\frac{1}{5}$	$\frac{1}{4}$	Turbid.
Fagus bulbus	1	1 (−)	1 (−)	1 (−)	$\frac{2}{3}$	$\frac{1}{5}$	$\frac{1}{2}$	Turbid, mucilaginous.
Cucumber (fruit, middle watery portion)	1	1 (−)	1 (−)	1 (−)	$\frac{2}{3}-\frac{3}{4}$	$\frac{2}{3}-\frac{3}{5}$	$\frac{2}{3}$	Slightly turbid.
Cucumber (basal portion)	1	1 (−)	1 (−)	1 (−)	$\frac{2}{3}$	$\frac{1}{3}-\frac{1}{4}$	$\frac{1}{3}$	Slightly turbid.
Apple	1	1 (−)	1 (−)	1 (−)	$\frac{2}{3}$	$\frac{1}{4}$	$\frac{1}{3}-\frac{1}{4}$	Clear.

The acidity is only given in a few cases; in the majority of plant juices it cannot be measured by a process of neutralizing on account of the formation of precipitates, and the presence of colour, opalescence, &c.

The above figures can only be interpreted in a very general way. When the plant juice forms a clear solution we must attribute its action on the fungal extract to the chemical substances contained in it. The high degree of retardation produced by the higher concentrations of lemon juice is obviously referable to its high acidity (cf. action of citric acid); in the others we must look upon the effect as being due to the combined action of acids, salts (probably including esters), &c. Where the plant juices are turbid, it is only possible with the data yet to hand to speculate as to the interpretation. Here the fine débris present may disturb the equilibrium of the colloidal enzyme in the solution, and may therefore be expected to increase the retarding effect of the plant juice.

The results with bean juice were very surprising in view of the great susceptibility of that plant, so it was necessary to examine this case in more detail. The expressed sap of bean leaf forms a deep green liquid, highly turbid. A portion of this turbidity can be removed by high-speed centrifuging, as also by passing through filter-paper. After the centrifuged liquid has stood for a short time (an hour) a further sediment can again be obtained by centrifuging, and so on. The crude juice can be driven through a Chamberland filter, and comes through free of turbidity. Soon, however, it is found that centrifuging causes a sediment in the filtered liquid. This process of precipitate formation is no doubt partly one of agglutination; it is also due in part to the oxidase action which takes place at the free surface, and which results in the formation of a black precipitate. In the crude juice, therefore, there is present a copious turbidity which cannot be got rid of by simple filtering. When the juice is boiled a very large precipitate is thrown down, and the juice now remains permanently clear. This boiled juice has a much smaller retarding effect than the crude juice.

Apart from the retarding action of substances contained in the bean extract upon the fungal extract, the former induces a marked change upon the potato discs themselves. This phenomenon has already been alluded to (p. 326, foot-note), but the present constitutes a much more striking manifestation. Discs of potato which have been kept for some time in bean juice possess diminished sensitiveness to the action of the fungal extract. Thus in one case a potato disc which had been kept for twenty-four hours in bean extract was found to be apparently unaffected by freshly prepared *Botrytis* extract after a two days' action, while a similar disc which had been kept for twenty-four hours in water was completely disintegrated by the same extract in sixty minutes. This 'hardening' effect is much greater in the case of the expressed sap of the leaves than in that of the stem; it is also more marked with potato than with turnip

discs. What the explanation of this phenomenon is it is at present impossible to say.

As participating in the abnormal retarding effect of bean juice upon the fungal extract, we have therefore to consider at least three factors:

1. The retarding action (properly so called) of chemical substances present in the bean juice.

2. The effect of the precipitate present.

3. The 'hardening' effect of the bean juice upon the potato tissue employed.

In comparing the experiments *in vitro* with what happens when the fungus invades the bean plant, it is to be noted that factors 2 and 3, which play a considerable part in the former, may not, and probably do not, have their counterpart in the latter.

The experiments above tabulated are very clear on one point—that the expressed sap of *Fegatella* has no special inhibitory action, but behaves in a manner quite comparable with the sap of potato, cucumber, &c. The marked resistance of the tissue of hepatics to the action of the fungal extract and to the fungus itself is not to be ascribed in any way to any specific anti-properties of the cell-sap.

H. Discussion of the Nature of the 'Lethal Principle'.

It has been shown in the foregoing that the macerating action of the fungal extract can be destroyed in various ways: by heat, by mechanical agitation, and by neutralization with alkali. The extracts so deactivated possess no lethal activity whatever. From microscopical investigation it is known that death of the cells takes place at a late stage in the process of disintegration of the cell-walls. The latter process is therefore the determining factor of the whole action. This dependence of lethal upon macerating activity may be explained in either of the two following ways:[1]

1. That both actions are due to the same substance or group of substances.

2. That the two actions are due to different substances, but the lethal substance is unable to reach the protoplast until the permeability of the cell-wall has been sufficiently increased by the action of the macerating substance.

In the absence of an exact knowledge of the diffusive capacity of the

[1] It might be suggested that no toxin exists in the fungal extract, but that it is produced as a result of the action of the extract upon the cell-wall. Experiment has shown that a fungal extract in which a quantity of well-washed, grated turnip tissue has been digested behaves similarly to standard extract as regards deactivation by heat and by neutralization with alkali. In other words, the hydrolysis products of cell-wall substance are not of toxic nature.

lethal principle in relation to the wall of the cells of susceptible tissue (a knowledge which obviously can be obtained only by indirect means) it is impossible to decide with complete certainty between the two hypotheses presented. Nevertheless from the following experiment the view that the two actions are brought about by the same substance is rendered the more probable.

In Section F it was shown that extracts which had not been completely deactivated by heat possessed lethal activity; and in Section G that both actions were stopped sharply when the extract was neutralized. If therefore the lethal and macerating substances are different, it is improbable that heat and percentage of alkali would affect both in the same degree. Killing of the cells should thus continue independently of the macerating action after a certain stage is reached, that is, when the permeability of the cell-wall has been sufficiently increased. Nevertheless it is found that if the macerating action is stopped, even at a very late stage, the killing effect is strongly retarded.[1] Such evidence is most readily interpreted according to the view that the lethal and macerating substances are identical.

If we accept the hypothesis that lethal and macerating actions are due to the same substance, death of the cells is to be looked upon either as due to the *direct* action of the macerating substance upon the protoplasmic membrane, or as the *indirect* result of the action upon the cell-wall, the phenomenon thus depending upon some special relationship between cell-wall and protoplasm. The former alternative predicates toxicity of the macerating substance; in the latter case death of the cells follows disintegration of the cell-walls in a manner that is not understood.

On the nature of the macerating substance little need be said. The present investigation bears out the conclusions of earlier workers that it is enzymic in nature. In the older literature it was known under the general name of 'cytase'; more lately it has been designated 'pectinase', from its

[1] It is impossible to stop the action of the extract by washing the partially disintegrated discs in water. The active principle remains adsorbed on the tissue, so that discs which have been taken from the active extract even at a comparatively early stage in the action and thoroughly washed in water are completely disintegrated in course of time. It is obvious, therefore, that no conclusions can be drawn from the behaviour of discs which have been taken from active extract and placed in extract which has been deactivated by heat. The only practicable method is to stop the macerating action by immersion of the discs in very dilute alkali $\left(\frac{n}{400}\right)$, after which they are transferred to an extract which has been rendered exactly neutral. In experiments with discs of Swede Turnip, it was found that up to and a little beyond the stage termed 'coherence gone', the above treatment considerably delayed the incidence of death. As the cell-walls have by this time undergone considerable disintegration, they cannot be conceived to be impermeable to the lethal principle present in the neutralized extract. We should therefore expect that discs at this advanced stage would show the killing effects as rapidly in the neutralized as in the ordinary extract. This, however, is not the case. That the discs in the neutralized extract do show killing after a longer or shorter time (depending on the stage at which they were removed from the active extract) is not surprising. It is probable that a certain amount of action on the protoplasmic membrane had already taken place when the discs were transferred to the neutralized extract.

pronounced action on the pectin constituents of the cell-wall, and more especially on the so-called calcium pectate of the middle lamella.

Whichever hypothesis be accepted as explaining the lethal action of the fungal extract, it is clear in any case that the chemical nature of the cell-wall is of fundamental importance in relation to the action of the fungal extract upon the cell. In all cases it has been found that if the cell-wall is disintegrated death of the cell ensues; if the cell-wall is not affected neither are the living contents of the cell. In other words, the nature of the cell-wall affords the key to the resistance of the particular tissue to the action of the fungal extract and therefore also of the fungus. In particular, certain experiments lead to the conclusion that there are important chemical differences between the cell-walls of higher plants and those of lower forms such as Hepaticae. These considerations point to the desirability of a more complete study of the hemicellulose (or pectin?) series of cell-wall constituents than has yet been attempted.

This investigation was undertaken at the suggestion of Professor V. H. Blackman, and has been prosecuted throughout under his guidance. It is with great pleasure that I take this opportunity of recording my indebtedness to him for many helpful suggestions and for his continued interest.

I. Summary.

1. A method of preparing a very powerful extract from the germ tubes of *Botrytis cinerea* is described (Sections C and E, *a*).

2. The action of the extract on plant tissue is twofold:

(*a*) Action on the cell-wall, leading to disintegration of the tissue.

(*b*) Action on the protoplast, producing death (Section E, *b*).

3. From microscopical investigation, death of the cells is seen to take place at a late phase of the process of disorganization of the cell-wall (Section E, *b*).

4. The extract may be deactivated by heating, by mechanical agitation, and by neutralization with alkali. Deactivation by any method leads also to the loss of the lethal power of the extract (Sections F and G).

5. Neither oxalic acid nor oxalates play any part in the toxicity of the extract. If any special lethal substance is present it must be of colloidal nature (Section F).

6. The only active substance in the extract appears to be the enzyme, which produces a macerating action mainly by solution of the middle lamella. The enzyme appears also to be responsible for the lethal action of the extract, the death of the cells being brought about either by direct action of the enzyme on the protoplastic membrane, or indirectly as a result of the action upon the cell-walls (Section H).

7. The ability of certain tissues to resist the action of the extract is dependent upon the special properties of their cell-walls (Sections E and G).

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